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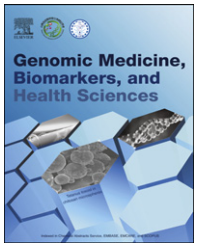

ORIGINAL ARTICLE

Preparation and evaluation of sucrose stabilized tetanus toxoid encapsulated into chitosan microspheres

Saravanakumar Arthanari [a], **Ponnusamy Renukadevi** [a,*], **Kavaretti Raju Mani** [b]

[a] *Biotechnology Centre, Anna University of Technology Coimbatore, Coimbatore, Tamilnadu, India*
[b] *DTP Section, Central Research Institute, Kasauli, Himachal Pradesh, India*

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Abstract Immunization is the most cost effective weapon for disease prevention in developing countries, and advanced molecular and genetic technologies are making new types of vaccines feasible. Here, the utility of both *in vitro* and *in vivo* methods to assess the release pattern of chitosan microspheres containing tetanus toxoid (TT) vaccine were evaluated. TT was stabilized and encapsulated in chitosan (TTCH) with a water-in-oil-in-water (W/O/W) multiple emulsion method using sucrose as a protein stabilizer. The TTCH prepared was smooth and spherical in shape with a diameter of around 10 μm. The *in vitro* release efficiency of TTCH was evaluated by differing stabilizer (sucrose) concentration (5%, 7%, 10% and 12% w/v) for a period of 70 days. The antigen release rates from the microspheres were determined by enzyme-linked immunosorbent assay. In these TTCH microspheres, a 10% w/v sucrose concentration gave good sustained antigen delivery for the period of 70 days. Based on the results of *in vitro* release, the *in vivo* studies were carried out using alum-adsorbed TT (from the Central Research Institute) as the standard. The antibody level was measured after 6 months, 9 months and finally, with one booster dose, after 12 months. In these *in vivo* studies, the TTCH antibody level rose up to 3.5 IU/mL of guinea pig serum; this compared with 2 IU/mL of guinea pig serum using the alum-adsorbed TT after 12 months with a second booster dose. The TTCH approach would be helpful to replace the existing adjuvant alum in the future.

* Corresponding author. Biotechnology Centre, Anna University of Technology Coimbatore, Jothipuram post, Coimbatore - 641047, Tamilnadu, India.
E-mail address: drrenukadevip@gmail.com (P. Renukadevi).

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Introduction

The majority of currently available vaccines require several booster doses to induce effective immunity and this results in significant compliance problems, particularly in the developing world. This is particularly true for immunization with several vaccines; for example, the triple vaccine against diphtheria, pertussis and tetanus, widely used in the Expanded Program on Immunization, demands administration of the vaccines at repeated intervals of 4–6 weeks, three times. These multiple injection patterns have limitations because a percentage of children who receive the first dose do not turn up for the second or third dose, so do not complete the immunization schedule and are not fully immunized.[1]

Currently, vaccine development is experiencing another wave of tremendous progress with the aid of modern immunology techniques and biotechnology. Various vaccine targets have been identified; alongside prophylactic disease resistance, vaccine development is ongoing for a number of cancers and AIDS.[2] Moreover, safe and efficacious vaccines have been developed, such as subunit, live-vector based and DNA vaccines.

Tetanus remains a major killer in developing countries, with more than 500,000 deaths per year from neonatal tetanus alone, largely due to the logistical difficulty of delivering the two to three doses of vaccine required to confer protection for pregnant women, whose immunity can be passively transferred to the fetus.[3]

Aluminum phosphate and aluminum hydroxide are the currently approved adjuvants for human vaccination and are widely used for vaccines at present.[4] In recent years, there have been various attempts to demonstrate new improved techniques to induce a higher level of immunogenicity following parenteral and/or oral administration.[5] One approach has been to use biodegradable polymer technology to simplify a sustained release antigen following parenteral administration, which, it is envisaged, will minimize the number of injections given in the normal regimen.[6] Our main aim in the present research is to develop a single-dose vaccine to reduce the multiple injections administered to infants during the first 3 months [the present tetanus toxoid (TT) vaccination schedule is one dose per month for the first 3 months]. With respect to our primary goal, the single-dose vaccine has been developed successfully and, upon further investigation, reduces even the need for booster doses. The original aim of the developed vaccine delivery was to develop a single dose to replace the initial multiple doses, not the booster dose; the booster dose is to raise the antibody level later on. Our current investigation is focused on reducing, or completely removing, the booster dose by increasing the initial load of antibody.

Chitosan is derived by the deacetylation of chitin, which is a polymer of D-glucosamine and *N*-acetyl-D-glucosamine. Chitosan is well-known for its hydrophilic, biocompatible, biodegradable and non-toxic properties.[7–9] Chitosan suspensions or micro- and nanoparticles have been reported to have immune stimulating activities, such as increasing accumulation and activation of macrophage and polymorphonuclear cells, promoting resistance to infections by microorganisms, and inducing cytokine response.[10] There are many advantages to using chitosan or chitosan microspheres for vaccine delivery. First, chitosan can open the intercellular tight junctions and favor the paracellular transport of macromolecules. Second, chitosan nano- and microspheres are suitable for controlled drug and vaccine release. Third, chitosan nano- and microspheres are most efficiently taken up by phagocytotic cells. Thus chitosan and its derivatives could induce strong systemic and mucosal immune responses against antigens.[8,10] Immunizations with various antigens co-administered with chitosan produce both systemic and local immune responses. In a phase I clinical study, intranasal immunization with an influenza vaccine formulated with soluble chitosan glutamate showed positive effects.[11]

Materials and methods

Chitosan (0.15 Pa.s viscosity grade, 80% deacetylation) was purchased from the Central Institute of Fisheries Technology (Cochin, India). Tween 80 and Span 80 were purchased from Fluka (Sigma Aldrich, Buchs, Switzerland). TT (molecular weight, 150 kDa), with a limit of flocculation (Lf) content of 1250/mL, and the standard tetanus antitoxin were received as gifts from the Central Research Institute (CRI, Kasauli, H.P., India). Sucrose and sodium tripolyphosphate (TPP) were purchased from Sigma Aldrich (St. Louis, MO, USA). All other chemicals and reagents used were of analytical grade.

Preparation of TT encapsulated chitosan microspheres

A water-in-oil-in-water (W/O/W) multiple emulsion technique was used to prepare chitosan microspheres by following the previously described procedure with slight modifications.[12] Four batches of microspheres were prepared by altering the stabilizer (sucrose) concentration (5%, 7%, 10% and 12% w/v). The dispersion phase was prepared by mixing 50 mL of linseed oil and 10% of Tween 80 at 1700 rpm for 10 minutes. To this, 3 mL of 1% stabilized chitosan gel were mixed separately to form a water-in-oil emulsion. To the above emulsion, 2 mL of alum-free TT (1250 Lf units/mL), which was previously stabilized with various concentrations of sucrose, was added and the stirring continued. After 2 hours, 2 mL of 5% w/v of TPP was introduced drop wise and stirring continued for a further 2 hours. Another 2 ml of TPP was then added and the stirring continued for another 2 hours. Finally, the suspension of microspheres was centrifuged at 15,000 rpm to remove the oil layer and the pellets were separated. The pellets were washed five times with 5 mL of toluene then washed three times with 5 mL of acetone. The microspheres were suspended in 10 mL of acetone and dried at room temperature. The dried microspheres were stored in sealed glass vials in a vacuum desiccator.

Physicochemical characterization of the microspheres

The morphological examination of the microspheres was performed using a scanning electron microscope (SEM; JSM-T

220 A, Jeol, Japan). The microspheres were mounted on to metal stubs using double sided carbon adhesive tape. After being vacuum-coated with gold palladium to a thickness of 200—300 Å, the microspheres were examined with the SEM at 15 or 10 kV (Fig. 1).

Estimation of TT content in chitosan microspheres

A weighed (10 mg) quantity of microspheres were powdered in an agate mortar and the TT was extracted with 6 mL of phosphate buffered saline (PBS) at pH 7.4, in screw-capped vials, by rotating the tubes head to tail in a hematology mixer for 7 days.[12] The total antigen content in the extract was determined by enzyme-linked immunosorbent assay (ELISA). The complete extraction was obtained by further treating the microspheres with 5% v/v hydrogen chloride in ethanol for another 2 days. Placebo microspheres (without the antigen) containing sucrose were used as a control.

In vitro release study

In vitro release studies of the prepared microspheres with various concentrations of sucrose (5%, 7%, 10% and 12% w/v) were carried out separately in conical flasks by taking 50 mg of microspheres and 50 mL of PBS with Tween 80 (pH 7.4) and incubating them at 37 °C on a constant shaking mixer.[12] The contents of the vial were withdrawn and centrifuged at 5000 rpm for 5 minutes at predetermined time intervals (Days 0, 2, 4, 8, 12, 16, 21, 28, 35, 42, 49, 56, 63 and 70). The TT concentrations in the supernatants were determined by ELISA. Placebo microspheres without the antigen were used as a control.

ELISA for TT

TT antigenicity was measured by ELISA as described by Johansen et al, with slight modifications.[13] Briefly, flat-bottom 96-well Nunc immuno microtiter plates were filled with 100 μL of 2 IU/mL of horse anti-tetanus immunoglobulin G in 0.05 M carbonate buffer of pH 9.6 overnight. The plates were washed three times with 300 μL of PBS containing 0.05% Tween 20. After this, a two-fold dilution series of sample and reference TT samples were prepared using PBS with 0.5% bovine serum albumin (BSA). The plates were held at room temperature for 24 hours followed by the addition of peroxidase-labeled sheep anti-TT serum (in PBS with 0.5% BSA) at room temperature for 2 hours. Finally, 100 μL of 0.2 mg/mL peroxidase substrate, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonate), in 100 mM sodium phosphate solution was added to the plates. The reaction was stopped by the addition of 50 μL per well of 5 N sulfuric acid. The plates were read on an ELISA reader after 30 minutes at a wavelength of 405 nm.

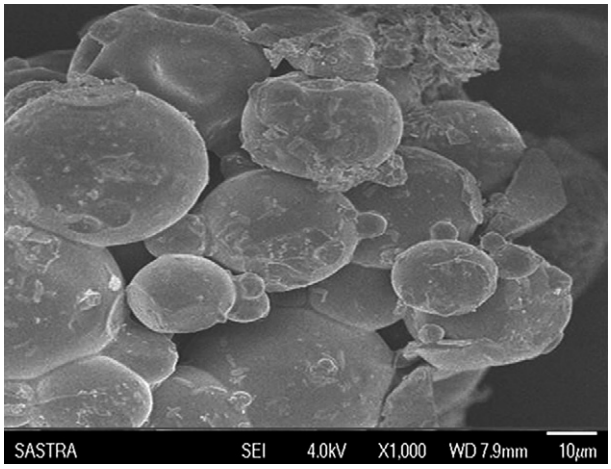

Figure 1 Scanning electron micrograph of the chitosan microspheres encapsulating the tetanus toxoid, prepared with 10% sucrose.

In vivo immunogenicity study

An ideal batch of chitosan microspheres encapsulated with TT (TTCH) was tested for immunizing potency and its antibody response to tetanus was measured in international units (IU/mL of serum). The vaccine combinations used for the immunization of the guinea pigs either with the booster dose or without the booster dose is categorized in Table 1. The animal studies were carried out based on the institutional animal ethical committee recommendations from CRI.

Each immunization combination (Table 1) was performed on nine guinea pigs (250—350 g). The booster dose, wherever indicated, was given not later than 4 weeks from the date of the primary immunization. Blood samples were collected about 2 weeks after the booster dose. The immune guinea pig sera were collected and tested in mice and guinea pigs for the measurement of tetanus antibodies. The potency test was conducted as per the standards of National Control Laboratories (CRI) and Indian Pharmacopoeia (IP, 1985).[14] Bleeding of the immunized guinea pigs was done by cardiac puncture using a 20-gauge needle. The blood samples were collected in sterile glass tubes and kept in a slanting position so as to allow the serum to ooze out of the clotted bottle. Then these tubes were centrifuged at 2000 rpm for 45 minutes. The supernatant immune serum from each tube was transferred to another sterile 5 mL vial using sterile Pasteur pipettes and numbered. The sera samples were inactivated at 55°C in a water bath for 20 minutes, before they were tested for tetanus antibodies by the antibody induction method.

Table 1 The various combinations used for *in vivo* immunogenicity study.

Combination	Primary immunization	Booster
1	TTCH	TTCH
2	TTCH	No booster
3	CRI[a]	TTCH
4	TTCH	CRI[a]
5	CRI[a]	CRI[a]
6	CRI[a]	No booster

CRI = Central Research Institute; TTCH = tetanus toxoid encapsulated in chitosan.
[a] Alum-adsorbed tetanus toxoid from the CRI.

Results

Compared with the other adjuvants, alum is relatively weak[15] and may not be able to sufficiently enhance the immune response toward a poorly immunogenic antigen. Physicochemical and morphological differences in alum vaccines owing to batch—batch variation and aging[16] can cause variability in the immune response that must be overcome by repeated injections, defeating the purpose of including the adjuvant in the first place. In many studies, aluminum-adsorbed vaccines did not show any advantage over soluble preparations for the booster or secondary response.[17—19]

Physical characterization of TT loaded chitosan microspheres

Four batches of TTCH microspheres were prepared and the effect of stabilizer concentration on *in vitro* release characteristics was evaluated. All the microspheres were prepared with a size range between 1 μm and 50 μm (Fig. 1). These size ranges were selected to define the role of macrophage uptake as an essential requirement for single point immunization.[20] In microspheres of 2—8 μm, more than 90% of the particles have a diameter less than 5 μm, so they will be taken up by antigen presenting cells[21] whereas with microspheres with a diameter of less than 2 μm there will be further enhanced cellular uptake due to the submicron size ranges of polymer particles.[22] With regard to the size range and percent of microspheres greater than10 μm, those prepared with 10% w/v of sucrose showed a more uniform distribution and optimum morphological characteristics, that is, no roughness, no porosity, no clumping and high sphericity (data not shown).

In vitro release study

The *in vitro* release of the antigen by TTCH microspheres stabilized with sucrose at different concentrations (5%, 7%, 10% and 12% w/v) was compared with microspheres prepared without the sucrose stabilizer. The TTCH microspheres with 10% w/v of sucrose showed a good release pattern, i.e., about 8—10 % of encapsulated antigen was released in the first 3—5 days. Similarly 7—9% of the antigen was released within the first day of incubation (Fig. 2 and Table 2) by the batch prepared with 7% w/v sucrose. All the remaining batches showed irregular or lower initial release. Among the batches prepared in chitosan microspheres, the batch prepared with 10% w/v sucrose showed the lowest release rate, which could be considered as an appropriate release pattern for mucosal immunization.

In vivo immunogenicity study

The level of tetanus-specific antibody present in the serum collected from guinea pigs immunized with TTCH and tetanus toxoid adsorbed on to aluminum phosphate gel (from CRI) is given in Table 3.

TTCH administered to nine guinea pigs as a primary immunization with one booster dose gave rise to immune sera containing a maximum antibody level of 2 IU/mL of guinea pig serum. When no booster was given, the level of antibody was found to be 1 IU/mL of guinea pig serum. The sera samples collected from guinea pigs primarily immunized with alum-adsorbed TT and given a booster dose of TTCH contained a maximum antibody level of 1.5 IU/mL of guinea pig serum. When the primary immunization was done with TTCH and alum-adsorbed TT given as a booster dose, the antibody level also rose, to 1.5 IU/mL of guinea pig serum. When the control-adsorbed TT was given for both the primary and booster inoculations, the antibody level in the immune sera was found to be 1.5 IU/mL of guinea pig serum. The antibody level was found to be only 1 IU/mL of guinea pig serum when no TT adsorbed booster given.

As per the I.P. requirement, all the combinations of primary immunization and booster dose passed the potency test—the minimum requirement as per the I.P. is that at

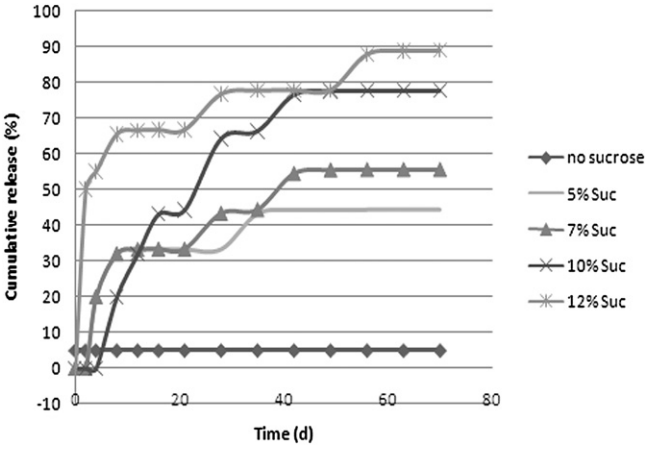

Figure 2 Release profiles of tetanus toxoid-loaded chitosan microspheres prepared with different concentrations of sucrose.

Table 2 *In vitro* release study of TTCH microspheres prepared by differing sucrose (stabilizer) concentration.

Day	Cumulative percentage release for each sucrose concentration			
	5%	7%	10%	12%
0	0	0	0	0
2	0	0	50.00	0
4	20.00	0	65.00	20.00
8	32.00	20.00	66.50	22.00
12	33.20	32.00	66.65	32.20
16	33.32	43.20	76.67	33.22
21	43.33	44.32	77.67	33.32
28	44.33	65.43	77.77	33.33
35	44.43	66.54	87.78	43.33
42	44.44	66.65	88.78	44.33
49	44.44	87.67	88.88	44.43
56	44.44	88.77	88.89	44.44
63	54.44	88.88	98.89	44.44
70	55.44	88.89	99.89	44.44

TTCH = tetanus toxoid encapsulated in chitosan.

Table 3 The antibody levels of immune guinea pig sera after first bleeding.

Combinations	Primary immunization	Booster	Antibody levels (IU/mL)					
			0.05	0.5	1	1.5	2	2.5
1	Chitosan (encap)	Chitosan (encap)	✓	✓	✓	✓	✓	—
2	Chitosan (encap)	No booster	✓	✓	✓	—	—	—
3	CRI[a]	Chitosan (encap)	✓	✓	✓	✓	—	—
4	Chitosan (encap)	CRI[a]	✓	✓	✓	✓	—	—
5	CRI[a]	CRI[a]	✓	✓	✓	✓	—	—
6	CRI[a]	No booster	✓	✓	✓	—	—	—

CRI = Central Research Institute; TTCH = tetanus toxoid encapsulated in chitosan.
[a] Alum-adsorbed tetanus toxoid from the CRI.

Table 4 The antibody level of immune guinea pig sera after 6 months of primary immunization.

S.no	Primary immunization	Booster	Antibody levels (IU/mL)								
			0.05	0.5	1	1.5	2	2.5	3	3.5	4
1	TTCH	TTCH	✓	✓	✓	✓	✓	✓	—	—	—
2	CRI[a]	CRI[a]	✓	✓	✓	✓	✓	—	—	—	—

CRI = Central Research Institute; TTCH = tetanus toxoid encapsulated in chitosan.
[a] Alum-adsorbed tetanus toxoid from the CRI.

least two thirds of the guinea pig sera contain a minimum antibody level of 0.05 IU/mL and at least one third of the guinea pig sera contains 0.5 IU/mL.

The highest level of antibody, 2.5 IU/mL of guinea pig serum, was found to be in the sera of guinea pigs immunized with TTCH as the primary and booster dose. When the control alum-adsorbed TT vaccine was given as the primary and booster dose, this gave rise to a maximum antibody level of 2 IU/mL of guinea pig serum. However, as per the I.P. requirement, the two combinations, 1 and 5 (see Table 1), passed the potency test. The results are shown in Table 4.

After 9 months, the same guinea pigs were again bled and their sera tested for specific antibody level. Guinea pigs vaccinated according to combination 1 still continued to have a maximum antibody level of 1 IU/mL of guinea pig serum. However, with combination 5, the maximum potency level was found to be 0.5 IU/mL of guinea pig serum. These findings show that, after a period of 9 months, there is gradual decline in the tetanus antibody levels. The results are shown in Table 5.

When given TTCH as the primary vaccination and booster (combination 1), the antibody level rose up to 3.5 IU/mL of guinea pig serum after the second booster dose. In combination 5, with the alum-adsorbed TT as the primary vaccination and booster, after the second booster the potency was found to be 2 IU/mL of guinea pig serum, which is not even comparable to the results from combination 1. The results are shown in Table 6.

Discussion

In our study, the main objective was to evaluate the suitability and potential of chitosan polymeric systems as adjuvants for the TT vaccine that are easy to deliver and elicit a long-lasting immune response. The actual size of the individual microspheres was in the range of 1—50 μm and a few of them were more than 50 μm. During the preparation of chitosan microspheres, it is necessary to use discordant solvents, which may degrade proteins. Exposing the microspheres to physiological environments may also destabilize the protein molecules.[1,3] It is thus necessary to stabilize the protein during both the encapsulation process and release from the microspheres. Therefore, the microspheres were co-encapsulated with a cheap potential protein stabilizer, sucrose. It has also been hypothesized that a protein stabilizer can shield the antigen from the

Table 5 The antibody level of immune guinea pig sera after 9 months of primary immunization.

S. nos	Primary immunization	Booster	Antibody levels (IU/mL)								
			0.05	0.5	1	1.5	2	2.5	3	3.5	4
1	TTCH	TTCH	✓	✓	✓	✓	✓	✓	—	—	—
2	CRI[a]	CRI[a]	✓	✓	✓	✓	✓	—	—	—	—

CRI = Central Research Institute; TTCH = tetanus toxoid encapsulated in chitosan.
[a] Alum-adsorbed tetanus toxoid from the CRI.

Table 6 The antibody level of immune guinea pig sera after 1 year of primary immunization with 2nd booster dose.

S.nos	Primary immunization	Booster	Antibody levels (IU/mL)								
			0.05	0.5	1	1.5	2	2.5	3	3.5	4
1	TTCH	TTCH	✓	✓	✓	✓	✓	✓	✓	✓	—
2	CRI[a]	CRI[a]	✓	✓	✓	✓	✓	—	—	—	—

CRI = Central Research Institute; TTCH = tetanus toxoid encapsulated in chitosan.
[a] Alum-adsorbed tetanus toxoid from the CRI.

organic solvent via preferential hydration of their surface, thus preventing protein-interface exposure to deleterious solvent effects.[23] The loading efficiency of chitosan microspheres containing 10% sucrose was increased from 80—90%, whereas the lowest TT payload was observed without sucrose. This could be due to inactivation of the TT at the water-oil interface (the first emulsification step).[24]

The TT release pattern from the chitosan microspheres stabilized with sucrose at different concentrations (5%, 7%, 10% and 12% w/v) was compared with microspheres without sucrose. TTCH microspheres without sucrose released only 5% of the loaded TT and no further release was observed over the following 70 days. Mechanical forces employed in the first emulsion might also cause protein structural perturbations, which often result in irreversible aggregation.[25] A potential approach to increase the TT concentration and loading during emulsification is by using a protein stabilizer (sucrose). This resulted in good stabilization upon TT encapsulation in and release from microspheres made using a W/O/W method.[26] The protein stabilizer prevents denaturation at the water-oil interface, which is reflected in an augmented cumulative percentage (88.88% for TTCH) release. In addition, sucrose has good solubility in aqueous media. The protein stabilizer (sucrose) concentration was directly proportional to the percentage release of TT from the microspheres. To this end, the higher concentration of sucrose (12%, w/v) increased the initial burst release of TT within 5 days. But the 10% sucrose mix increased the sustained release of TT up to 70 days as compared to 12% sucrose. In conclusion, 10% w/v sucrose was found to be a specific, sensitive, reproducible and stable antigen during the encapsulation and release system.

In the immunogenicity study, microspheres with 10% w/v sucrose were used. In this study, the levels of antibody were equal or higher when measured 6 months, 9 months and 12 months after primary immunization. The results of *in vivo* studies indicate that TTCH microspheres have potential application in the field of vaccine delivery and could be an appropriate choice for the development of a single-dose vaccine against tetanus in the future.

Conclusions

In the present studies, we have selected chitosan as a suitable adjuvant, and 10% w/v of sucrose as a stabilizer lead to better *in vitro* release characteristics. The potency of TTCH was tremendously increased after 1 year with a second booster dose when compared to standard alum-adsorbed TT (CRI). Further studies are required to investigate in greater detail the use of chitosan and its derivatives for their pharmaceutical applications.

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